PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 21/04, C12P 19/34, C12Q 1/18, 1/37, 1/68

A1

(11) International Publication Number: WO 95/34574

(43) International Publication Date: 21 December 1995 (21.12.95)

(21) International Application Number: PCT/US95/07755

(22) International Filing Date: 16 June 1995 (16.06.95)

(30) Priority Data:
08/261,068 16 June 1994 (16.06.94) US

(71) Applicant *(for all designated States except US)*: MICROPROBE CORPORATION [US/US]; Suite 104, 1725 - 220th Street, S.E., Bothell, WA 98021 (US).

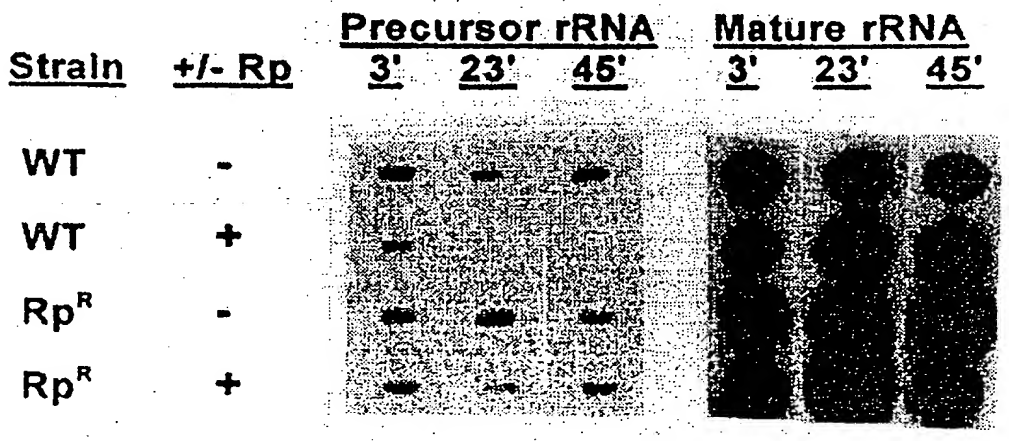
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BRITISCHGI, Theresa, B. [US/US]; 17514 Densmore Avenue, North, Seattle, WA 98133 (US). CANGELOSI, Gerard, A. [US/US]; 314 Northwest 72nd Street, Seattle, WA 98117 (US).

(74) Agents: WEBER, Kenneth, A. et al.; Townsend and Townsend and Crew, Steuart Street Tower, One Market Plaza, San Francisco, CA 94105-1492 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RAPID AND SENSITIVE DETECTION OF ANTIBIOTIC-RESISTANT MYCOBACTERIA USING OLIGONUCLEOTIDE PROBES SPECIFIC FOR RIBOSOMAL RNA PRECURSORS



(57) Abstract

The invention relates to methods and oligonucleotide probe compositions useful for determining antibiotic resistance in *Mycobacteria*. Included are methods for freeing intact precursor ribosomal RNA from mycobacterial cells and for assaying the levels of pre-rRNA in the cells. Also claimed are methods useful in discovering new anti-mycobacterial therapeutic agents.

ENSDOCID: <WO___9534574A1_I_>

RAPID AND SENSITIVE DETECTION OF ANTIBIOTIC-RESISTANT MYCOBACTERIA USING OLIGONUCLEOTIDE PROBES SPECIFIC FOR RIBOSOMAL RNA PRECURSORS

BACKGROUND OF THE INVENTION

Technical Field of the Invention

This invention relates to methods and oligonucleotide probes for use in the detection and identification of mycobacteria. The invention is particularly useful for detecting antibiotic resistant mycobacterial isolates. The oligonucleotide probes consist essentially of a segment of nucleic acid capable of selectively hybridizing under hybridizing conditions to the mycobacterial ribosomal RNA. Methods for detection, as well as diagnostic kits for the assay of these bacteria, are also disclosed.

Rapid identification of microbial pathogens has long been an important goal of diagnostic technology. A new challenge is posed by the spread of antibiotic resistance. In addition to identifying the pathogenic species, the clinician must now affirm the potential efficacy of standard antimicrobial treatments early in the treatment of each case. Delays and erroneous results associated with conventional antibiotic susceptibility tests frequently lead to the administration of ineffective treatments, which in turn leads to complications, added costs, and poor outcomes.

Antibiotic-resistant *Mycobacterium* strains present one of the most difficult diagnostic challenges. The genus *Mycobacteria* is composed of Gram-positive, acid-fast bacteria. Taxonomically, the *Mycobacterium* genus is divided into descriptive groups based on growth rate and pigmentation. Among these groups are the photochromogenic (pigmented) slow growers (Group I), the scotochromatic slow growers (Group II), the nonchromogenic slow growers (Group III), and the rapid growers (defined as maturing in less than one week) (Group IV) [Sommers and Good (1985) In E.H. Lennette *et al.* (Eds.), *Manual of clinical microbiology*, 4th ed., American Society for

BNSDOCID: <WO___9534574A1_I_>

oligonucleotide probe would detect species-specific DNA or RNA sequences, while another probe detects common antibiotic resistance genes. However, this method is useful only for known antibiotic resistance genes. Many different mechanisms can result in antibiotic resistance; many of these are not well understood. For example, resistance of *M. tuberculosis* to isoniazid can arise through mutations that reduce the expression of the catalase-peroxidase (*katG*) gene, or through separate mutations that enhance the expression of the *inhA* gene [Zhang *et al.* (1992) *Nature* 358: 591-593]. Similarly, resistance to rifampin can arise through any of a large number of missense mutations scattered over 1000 bases of the *rpoB* gene [Telenti *et al.* (1993) *The Lancet* 341: 647-650]. Because of such diversity, genetic probing techniques are frequently less useful than phenotypic tests for antibiotic effectiveness, such as the culture or BACTEC methods, which can detect resistance regardless of its genetic basis.

Each of the above methods of detecting antibiotic-resistant mycobacterial isolates lacks one or more of the following criteria necessary for a routine diagnostic assay: 1) cost (inexpensive); 2) batch capability; 3) speed (hours, not days or weeks); 4) sensitivity; 5) specificity; 6) ability to both identify the mycobacterium species and to determine whether the mycobacterium is resistant to antibiotics in one test; and 7) ability to detect drug-resistant mycobacteria regardless of the genetic basis for resistance. Consequently, there is a need in the art for alternative detection methodologies that are both fast and sensitive to a wide range of mycobacteria.

Information Disclosure

Previously described methods for identifying the species to which cells of a mycobacterial sample belong have serious shortcomings. Many species are not discernable by classical techniques, such as hybridization using oligonucleotide probes specific for the mature 16S rRNA. For example, a commonly used DNA probe test for identification of *Mycobacterium* species, the Gen-Probe Rapid Diagnostic System,

BNSDOCID: <WO___9534574A1_I_>

on the resulting pre-rRNA molecules. The tails are removed from this pre-rRNA during the secondary steps in rRNA processing to yield the mature rRNA [*see, e.g.*, King *et al.* (1986) *Microbiol. Rev.* 50: 428-451]. Because the first processing step occurs much faster than the second, the steady-state levels of pre-rRNA molecules are generally much higher than the levels of the primary transcripts. Thus, pre-rRNA is often detectable experimentally, whereas the primary transcripts are not.

Previous studies, mostly using *E. coli* as a model system, have demonstrated that pre-rRNA copy number rapidly decreases in sensitive cells that are treated with certain antibiotics that inhibit RNA synthesis [Srivastava *et al.* (1990) in *The Ribosome: Structure, Function, and Evolution*, W.E. Hill *et al.* (Eds.), American Society for Microbiology, Washington DC, pp. 426-434; King *et al.* (1983) *J. Biol. Chem.* 258: 12034-12042]. Presumably, *de novo* pre-rRNA synthesis is inhibited while maturation proceeds.

Mycobacterial rRNA undergoes processing similar to that of other bacteria. However, prior to the present invention, pre-rRNA had never been detected in mycobacteria, despite many attempts. Researchers have advanced several hypotheses to explain this failure. One hypothesis is that the waxy cell wall of mycobacteria is unusually difficult to rupture. To detect pre-rRNA, one must first free the pre-rRNA from the cells. Methods sufficiently harsh to lyse the waxy mycobacterial cell wall might cause degradation of the cellular pre-rRNA.

Another hypothesis advanced to explain why pre-rRNA had never been detected in mycobacteria is that mycobacteria have a very low copy number of precursor rRNA. Ji *et al.*, after failing to detect mycobacterial pre-rRNA, hypothesized that mycobacterial pre-rRNA is processed rapidly after transcription. Thus, the ratio of pre-rRNA to mature rRNA would be inversely proportional to the doubling time of a bacterium [Ji *et al.* (1994) *J. Infect. Diseases* 169: 305-312]. A third hypothesis is that the extensive secondary and

BNSDOCID: <WO___9534574A1_I_>

detergent, and the cells are heated to between about between 75°-99°C until the cells are lysed.

The invention also includes methods for detecting pre-ribosomal RNA (pre-rRNA) in cells of a mycobacterial sample. The methods comprise treating the cells by enzymatic or mechanical means to expose the cell membrane to lysis reagents, contacting the cells with a lysis reagent under conditions that release but do not degrade the pre-rRNA, and detecting the pre-rRNA using at least one oligonucleotide probe.

Also claimed are methods for determining whether cells of a mycobacterial sample are sensitive to an antimicrobial agent. The mycobacterial cells are incubated in the presence of the antimicrobial agent, after which the cells are treated by enzymatic or mechanical means to expose the cell membranes to lysis reagents. The cells are then contacted with a lysis reagent under conditions that release but do not degrade the mycobacterial pre-rRNA. The mycobacterial pre-rRNA is detected using an oligonucleotide probe. Sensitivity to the antimicrobial agent is indicated by an increase or a decrease in pre-rRNA levels for mycobacterial cells exposed to the antimicrobial agent compared to mycobacterial cells not exposed to the antimicrobial agent.

Oligonucleotide probes are also claimed. The claimed probes are between about 10 to 100 nucleotides in length and are capable of selectively hybridizing, under hybridizing conditions, to a region of a mycobacterial pre-rRNA molecule that is not present in a mature mycobacterial rRNA molecule.

The present invention also includes a device for detecting pre-rRNA in a mycobacterial sample. The device consists of an oligonucleotide probe, as described above, that is immobilized on a solid support. Also claimed is a kit for detecting pre-rRNA in a mycobacterial sample. The kit contains a device as described above and a second oligonucleotide probe that is between about 10 to 100 nucleotides in length and is capable of selectively hybridizing, under hybridizing conditions, to a region of a

BNSDOCID: <WO___9534574A1_I_>

Values for treated cultures are the means for three experiments.

Figure 3. Diagram showing the elements of an rRNA hybridization "sandwich" assay.

Figure 4. Results of a chemiluminescent sandwich assay for *M. tuberculosis* pre-16S rRNA. The experiment was performed as described in Example 4.

Figure 5. Assessment of mycobacterial response to rifampicin and ciprofloxacin. Replicate cultures were exposed to the antibiotics or left untreated, and samples taken as described in Example 7. A., Slot blot hybridization assays for pre-16S rRNA detected by probe MTB030, and combined precursor and mature 16S rRNA detected by probe UP041. Time in hours after addition of antibiotics is shown to the left. N, no treatment; Rp, rifampicin; Cp, ciprofloxacin. B., Culture density (optical density at 600 nm) of the same cultures at the same time points. Squares, no treatment; circles, rifampicin; triangles, ciprofloxacin. Identical results were obtained in replicate experiments.

Figure 6. Comparison of (A) pre-16S rRNA detected by probe MTB030, and (B) the DNA sequence encoding pre-16S rRNA detected by complementary probe MTB030r, in slot blot hybridization assays. Replicate cultures were exposed to rifampicin (+Rp) or left untreated as described in Example 7. Time in hours after addition of the antibiotic is shown to the right.

DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention relates to methods for characterizing *Mycobacteria*, both in terms of identifying particular species and determining whether a particular isolate is resistant to an antibiotic. The methods combine the comprehensive sensitivity of phenotypic tests for antibiotic susceptibility with the speed and species specificity of oligonucleotide probe methods. Also included in the invention are oligonucleotide probes suitable for use in the claimed methods, and methods for freeing precursor ribosomal RNA from mycobacterial cells.

BNSDOCID: <WO___9534574A1_I_>

conventional means to culture mycobacterium is Good and Mastro, *supra*., and Heifets, *supra*.

BNSDOCID: <WO___9534574A1_I_>

The cells can be concentrated prior to lysis. The specific means are not critical and include low speed centrifugation and filtration means. The concentrated cells are then resuspended into a low salt buffer comprising a divalent chelator and having a pH of about 4.5 to 8.0. The buffer can optionally contain a reducing agent such as mercaptoethanol, dithiothreitol, or dithioerythritol, or a combination thereof. Suitable chelators include ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis(β-amino-ethyl ether)-tetraacetic acid (EGTA), and ethylene diimino dibutyric acid (EDBA).

Suitable buffers include, but are not limited to, brucine tetrahydrate, 4-(2-hydroxyethyl)-1-piperazinepropane sulfonic acid, tris(hydroxymethyl) aminomethane, N-tris(hydroxy-methyl) methylglycine, glycinamide, N,N-bis(2-hydroxyethyl) glycine, N-tris(hydroxymethyl)methyl-2-aminopropane sulfonic acid, N-glycyl-glycine, histidine, boric acid, pyrophosphoric acid, ethanolamine, glycine, trimethylamine, cyclopetanetetra-1,2,3,4-carboxylic acid, carbonic acid, 3-cyclohexylamino-1-propanesulfonic acid, EDTA, methylamine, dimethylamine, ethylamine, triethylamine, diethylamine, ascorbic acid, and phosphoric acid, sodium acetate, and Tris.

The cells are then treated to perturb or compromise the integrity of the cell wall. This exposes the cell membranes to the lysis reagent, which is added in a succeeding step. In one preferred embodiment, the mycobacterium cell wall is treated by enzymatic digestion. The resuspended cells are treated with a combination of lysozyme (5-20 mg/ml) and an endoprotease such as PRONASE™ or proteinase K (0.5 to 0.05 mg/ml) (Sigma Chemical Co., St. Louis MO). "Lysozyme" refers to enzymes that attack bacterial cell walls by hydrolyzing the β(1-4) linkages between N-acetyl-D-muramic acid and 2-acetylamino-2-deoxy-D-glucose residues. Lysozyme also acts on chitin. "Protease" refers to enzymes that catalyze the breakdown of proteins. "PRONASE™" is a nonspecific protease isolated from *Streptomyces griseus*.

BNSDOCID: <WO___9534574A1_I_>

guidelines, one of skill using routine titration experiments can optimize the lysis conditions for any mycobacterium at any concentration. Typically, the suspension will be incubated for about five minutes or longer at 85°C.

The degree of cell lysis can be determined by the detection of released nucleic acid. The methods for such detection are not critical. The hybridization assays described herein are suitable for detecting release of nucleic acids.

Nucleic Acid Hybridization Methods

A significant advantage of the claimed invention is that it incorporates rapid and convenient sample-handling and detection methods. In one embodiment, hybridization assays are conducted directly on bacterial lysates, without the need to extract the nucleic acids. This eliminates several steps from the sample-handling process and speeds up the assay.

To perform such assays on crude cell lysates, a chaotropic agent is typically added to the cell lysates prepared as described above. The chaotropic agent stabilizes nucleic acids by inhibiting nuclease activity. Furthermore, the chaotropic agent allows sensitive and stringent hybridization of short oligonucleotide probes to RNA at room temperature [Van Ness and Chen (1991) *Nucl. Acids Res.* 19: 5143-5151]. Suitable chaotropic agents include guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, and cesium trifluoroacetate, among others. Typically, the chaotropic agent will be present at a final concentration of about 3 M. If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

Alternatively, one can purify the pre-rRNA prior to probe hybridization. A variety of methods are known to one of skill in the art (*e.g.* phenol-chloroform extraction, IsoQuick™ extraction (MicroProbe Corp., Bothell WA), and others). Pre-hybridization purification is particularly useful for standard filter hybridization assays (as described

BNSDOCID: <WO___9534574A1_I_>

Hybridization Conditions

Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2% detergent, such as sodium dodecylsulfate, or between 0.5-20mM EDTA, FICOLL™ (Pharmacia Inc.) (about 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/ml, fragmented nucleic DNA, *e.g.*, calf thymus or salmon sperm DNA, or yeast RNA, and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as polyethylene glycol, anionic polymers such as polyacrylate or polymethylacrylate, and anionic saccharidic polymers, such as dextran sulfate.

In one preferred embodiment, nucleic acids from GuSCN-lysed bacteria can be immobilized directly onto nitrocellulose or Nytran, and hybridized with the appropriate probe. The GuSCN-lysate is diluted with buffer containing formaldehyde, slotted to nitrocellulose and heated at 80°C to denature the nucleic acids. The hybridization solution comprises about 2 to 4M GuSCN, preferably 3M, about 0.01 to 0.1M Tris (pH range about 6.0 to 8.5), a detergent such as sodium dodecyl sulfate in concentrations of about 0.1 to 5% (w/v), and about 0.01 to 0.1M EDTA. Other additives may also be included such as carrier DNA or RNA, or protein such as bovine serum albumin or gelatin. Stringency of the hybridization solution can be adjusted by the addition of about 0 to 10% formamide, usually 5%. The hybridization is typically carried out for between 15 minutes and 16 hours, with about 1 hour being optimal. Hybridization and wash conditions are as described in Van Ness and Chen, *supra.*; Van Ness *et al.* (1991) *Nucl. Acids Res.* 19: 3345-3350; Cangelosi

BNSDOCID: <WO___9534574A1_I_>

rRNA or pre-rRNA that has formed a hybridization duplex with the immobilized nucleic acid probe on the solid support. This second probe is typically labelled. The presence of pre-rRNA is then determined in accordance with the label being used. It should be noted that the second probe can be added simultaneously with the test sample to the hybridization assay. In addition, the second probe can hybridize to either a conserved or to a hypervariable region of the rRNA or pre-rRNA. Preferred are the probes derived from conserved regions of the ribosomal RNA or pre-rRNA that have minimal secondary and tertiary interactions. The advantage of such probes is that the hybridization can be carried out without the additional step of heat denaturing the nucleic acid. A general reference for various detection methods can be found in Hames, B.D. and Higgins, S.J., *Nucleic Acid Hybridization*, IRL Press, Oxford, 1985. References for sandwich assay with DNA probes are Dunn and Hassell (1977) *Cell* 12: 23-26; and Ranki *et al.*, U.S. Patent No. 4,486,539.

Hybridization techniques are generally described in *Nucleic Acid Hybridization: A Practical Approach*, Hames and Higgins, Eds., IRL Press, 1987; Gall and Pardue (1969) *Proc. Natl. Acad. Sci., USA*, 63: 378-383, and John, Burnsteil and Jones (1969) *Nature* 223: 582-587. As improvements are made in hybridization techniques, they can readily be applied. One such improvement is the subject of Serial No. 130,754, our docket 11652-5, filed on December 9, 1987, incorporated herein by reference, which relates to the use of ultrasonic energy to enhance the rate of hybridization. This is an optional step which does not influence the specificity of the probes described herein.

The amount of labeled probe which is present in the hybridization solution may vary widely, depending upon the nature of the label, the amount of the labeled probe which can reasonably bind to the cellular target nucleic acid, and the stringency of the hybridization medium and/or wash medium. Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be

BNSDOCID: <WO___9534574A1_I_>

of parameters is more important than the absolute measure of any one.

Assay test protocols for use in this invention are those of convention in the field of nucleic acid hybridization, and include both single phase, where the target and probe polynucleic acids are both in solution, and mixed phase hybridizations, where either the target or probe polynucleotides are fixed to an immobile support. The assay test protocols are varied and are not to be considered a limitation of this invention. A general review of single phase hybridization can be had from a reading of *Nucleic Acid Hybridization: A Practical Approach*, Hames and Higgins, eds., IRL Press, 1985; and *Hybridization of Nucleic Acids Immobilized on Solid Supports*, Meinkoth and Wah (1984) *Analytical Biochemistry*, pp. 238, 267-284. Mixed phase hybridizations are preferred.

Cultured colonies of the mycobacteria can be assayed using colony hybridization techniques, wherein the mycobacteria are plated and adsorbed onto a filter, after which the colonies are lysed and the pre-rRNA freed from the cells by the methods described herein. The filters are then exposed to the oligonucleotide probes (Grunstein and Hogness 1979 *Methods of Enzymology*, Ed. Ray Wu, Vol. 68, pp. 379-409; and *Proc. Natl. Acad. Sci. U.S.A.* 72: 3961-3965 (1975).

Regardless of the assay test protocol being used, the mycobacterial nucleic acids are to remain in contact with a hybridization solution at a moderate temperature for an extended period of time. In single phase assays, the double-stranded duplexes may be separated from single-stranded nucleic acid by S_1 nuclease digestion followed by precipitation of duplex molecules, or by selective binding to hydroxyapatite. In mixed phase assays, the support-immobilized nucleic acids are introduced into a wash solution having analogous concentrations of sodium chloride, buffers, and detergent, as provided in the hybridization solution. The time period for which the support is maintained in the wash solution may vary from several minutes to three hours or more.

BNSDOCID: <WO___9534574A1_I_>

conjugating enzymes to oligonucleotides are known. *See, e.g.*, *Nucleic Acid Res.*, 14: 6115-6128 (1986) and *Nucl. Acid Res.*, 15: 5275-5287 (1987).

Oligonucleotide Probes

Oligonucleotide probes that are useful in the claimed methods comprise a further aspect of the invention. The claimed oligonucleotide probes are specific for ribosomal RNA precursor (pre-rRNA) molecules of mycobacterial species. Typically, the probes are between 10 and 100 nucleotides in length, and are capable of hybridizing substantially, under hybridizing conditions, to a region of a mycobacterial pre-rRNA molecule that is not present in a mature mycobacterial rRNA molecule. By "substantially", it is meant that under standard hybridization conditions of high stringency, percent hybridization can be shown to exceed 50% of the hybridization between perfectly complementary nucleic acid fragments. The probes selectively hybridize to pre-rRNA molecules, meaning that under standard hybridization conditions, the probes do not substantially hybridize to mature rRNA molecules.

Precursor rRNA sequences are known to have extensive secondary structure (intramolecular strand hybridization). This secondary structure is tenacious and chemical or heat denaturation will only temporarily disturb it. The secondary structure returns when the conditions are amended to allow probes to hybridize. While nucleotide sequence analysis has led researchers to hypothesize that single-stranded "open" regions exist in *E. coli* pre-rRNA molecules [Srivastava and Schlessinger (1990) In *The Ribosome Structure, Function and Evolution*, Hill *et al.*, eds., American Society for Microbiol., Washington DC, pp. 426-434]. These putative open regions are thought to have minimal secondary and tertiary interactions with other nucleotides.

The presence of open regions must be confirmed empirically. Researchers have postulated, again based on sequence analysis, that mycobacterial pre-rRNA also has single- and double-stranded regions [Kempsell *et al.* (1992) *J.*

BNSDOCID: <WO___9534574A1_I_>

substantially hybridize, under standard hybridization conditions, to the complementary pre-rRNA region of the mycobacterial species for which the probe is specific but do not substantially hybridize to pre-rRNA of a different species. Standard hybridization conditions are of high stringency. Typically, these probes hybridize to hypervariable regions of the pre-rRNA, which are regions having a nucleotide sequence that is peculiar to a particular mycobacterial species or type.

Also claimed are certain species-specific oligonucleotide probes that selectively hybridize to the mature rRNA molecules of various mycobacterial species. Importantly, these claimed species-specific oligonucleotides that hybridize to mature rRNA molecules function well in hybridizations carried out under non-denaturing hybridization conditions, even though their target sequences are reported to be double-stranded under such conditions [Kempsell *et al.* (1992) *J. Gen. Microbiol.* 138: 1717-1727]. Table 4 lists target sequences having these properties that are suitable for various *Mycobacteria* species. Knowing the sequences of target regions that are effective for *M. tuberculosis*, one of skill can identify the homologous sequences from mature rRNA of other mycobacterial species by performing a nucleotide sequence alignment. *See, e.g.*, Rogall *et al.* (1990) *Int. J. Syst. Bacteriol.* 40: 323-330.

The use of species-specific probes enables the clinician to identify a mycobacterial species and determine its antibiotic susceptibility even in the presence of other species. Thus, one can make the determination on a primary broth culture of a patient isolate. Colony isolation to obtain a pure culture is not required. If an *in vitro* RNA amplification method such as 3SR is employed, the experiment can be carried out directly on patient samples. Both of these methods represent a significant advance over the time-consuming methods of the prior art.

For example, it is clinically important to differentiate the *M. tuberculosis* complex from species in the *M. avium* complex. Making this differentiation by virtue of

BNSDOCID: <WO___9534574A1_I_>

BNSDOCID: <WO__9534574A1_I_>

Table 2: Target and probe sequences for oligonucleotides specific for *Mycobacterium tuberculosis* pre-rRNA molecules

PROBE	DESCRIPTION	TARGET SEQUENCE (5'-3')	PROBE SEQUENCE (5'-3')
MTB015	5' pre-23S, -1	UUCUUUGUGCAAUAUUUUUUUUCUUUGGUUUUU GUUGUG	CACAACAAAAACCAAAGAATATTGCACA AAGAA
MTB016	5' pre-23S, -35	ACGCTGCCGGCTAGCGGTGGCGTG	CACGCCACCGCTAGCCGGCAGCGT
MTB017	5' pre-23S, -51	TTGCGAGCATCAATGGATACGCTGCC	GGCAGCGTATCCATTGATGCTCGCAA
MTB018	5' pre-16S, -58	TTGTCGGGGGGCGTGGCCGTTTG	CAAACGGCCACGCCCCCCACAA
MTB024	3' pre-16S, +96	GGCCACCAACACACTGTTGGGTCCTGAGGC	CCTCAGGACCCAACAGTGTGTTGGTGGC
MTB027	3' pre-16S, +1	AAGGAGCACCAGCAAAACGCCCC	GGGGCGTTTTGCTGGTGCTCCTT
MTB030	5' pre-16S, -1	CCCUUUUCCAAAGGGAGUGUUUGGG	ACCCAAACACTCCCTTTGGAAAAGGG
MTB035	3' pre-16S, +75	GGGUGCAUGACAAGAAAGUUGGCCA	TGGCCAACTTTGTTGTCATGCACCC

Descriptions:

1) 5' or 3' refers to whether the target is on the 5' or 3' tail of the pre-rRNA;

2) pre-16S or pre-23S identifies the rRNA upon which the target sequence is located; and

3) Negative numbers refer to the number of nucleotides upstream of the mature rRNA 5' terminus that corresponds to the 3' end of the target sequence, and positive numbers refer to the number of nucleotides downstream of the mature rRNA 3' terminus that corresponds to the 5' end of the target sequence

SUBSTITUTE SHEET (RULE 26)

Table 3 (cont): Target sequences for oligonucleotide probes specific for pre-rRNA molecules of various *Mycobacteria* species

M. leprae:

1) 5'AAGGAGCACCACGAAAAACACUCUAA3'
2) 5'GGGUGCGCAACAGCAAAUAUCCA3'
3) 5'CCAGACACACUGUUGGGUCCUGAGGC3'
4) 5'UUGCGAGCAUCUAAAUGGAUGCGUUGUC3'
5) 5'GCGUUGUCAGUUAUGUAGUGGUGGCGU3'
6) 5'AUUCAUUGAAAAUGUGUAAUUUUCUUCUUUGGUUUUGUG3'
7) 5'UGUGUGUAGGUGUAGUUUAUUA3'
8) 5'CUAGAAAUUGAAAAUUUCGUCUAGUUAUUGAUGGAGUU3'

M. simiae:

1) 5'AAGGAGCACCACGAGAAACACUCC3'
2) 5'GGGUGCACAACAACAGGCAAUCGCCA3'
3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3'

M. paratuberculosis:

1) 5'UGUGUGGGUAUGGCAA3'
2) 5'CUGAUUUGAAAUUCACCUCGCUGCGCGAGGAGAU3'

M. marinum:

1) 5'UGUGAGGGAGUAGUCGUU3'
2) 5'CUGAUUGCGAAUUCACCUCGUUAUCGAGGGGUU3'

M. habana:

1) 5'UGUGUAGGUAUGGUCGU3'
2) 5'CAGAUUAUCUCUGAUUCGAAUCCACCUCGUUGAUCGAGGAGAU3'

BNSDOCID: <WO___9534574A1_I_>

Probes can have sequences complementary to the target sequences listed in Tables 2 to 4, or their equivalent. One of skill will recognize that oligonucleotide probes complementary to specific subsequences of the target regions, but not to the entire target region, will also function in the claimed assays so long as such probes substantially hybridize to the target regions.

The probes can be used by themselves as a single unit for binding, or the probes may be comprised of additional sequences not having the capacity to bind to pre-rRNA. Probes comprising more than the short sequences, as offered in Tables 2 to 4, may have repeating units of the same sequence (*e.g.*, concatemers of a sequence), a mixture of different sequences specific to one species of mycobacteria, and even a mixture of sequences that may be specific to one or more mycobacterial species.

If such probes are to contain concatemers of short sequences, said long probes will display the high hybridization specificity inherent in a "short" probe containing, for example, only 20 nucleotides. This concatemeric probe sequence could be contained within the cloning vector sequences and would have the structure given by the formula below.

Alternatively, the oligonucleotide probe can comprise a concatemer that has the formula [X-Y-Z]n, wherein:

a) X is a sequence of 0 to 100 nucleotides or nucleotide analogs that are not complementary to conserved or non-conserved regions of mycobacterial pre-rRNA;

b) Y is a sequence of 10 to 100 nucleotides or nucleotide analogs that are capable of hybridizing under hybridizing conditions to a region of the mycobacterial pre-rRNA that is not present in mature rRNA, such that Y may also comprise subsequences that are capable of hybridizing under hybridizing conditions to pre-rRNA of only one species of mycobacteria, of two species of mycobacteria, or to pre-rRNA of three or more species of mycobacteria;

c) Z is a sequence of nucleotides the same as or different from X, such that nucleotides or nucleotide analogs

3NSDOCID: <WO___9534574A1_I_>

or into a vector containing the SP6 promotor (*e.g.*, generation of single-stranded RNA using SP6 RNA polymerase), and transformation of a bacterial host. The oligonucleotide probes can be purified from the host cell by lysis and nucleic acid extraction, treatment with selected restriction enzymes, and further isolation by gel electrophoresis.

Oligonucleotide probes can be chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short probes of between 15 and 50 bases. For this invention, it is preferred to chemically synthesize short DNA probes using the Model 380B DNA Synthesizer from Applied Biosystems, Foster City, California, using reagents supplied by the same company. Probes can be comprised of the natural nucleotide bases or known analogs of the natural nucleotide bases, including those modified to bind labeling moieties. Oligonucleotide probes that comprise thionucleotides, and thus are resistant to nuclease cleavage, are also suitable.

Probes can be labeled by any one of several methods typically used to detect the presence of hybrid polynucleotides. The most common method of detection is the use of autoradiography using probes labeled with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P, or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability, and half lives of the selected isotopes. Other labels include ligands which bind to antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The choice of label dictates the manner in which the label is bound to the probe. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes, for example, by using DNA synthesizers, by nick translation with DNA polymerase I, by

BNSDOCID: <WO___9534574A1_I_>

Oligodeoxynucleotide-Alkaline Phosphatase Conjugates and Their Use as Hybridization Probes. *Nuc. Acids. Res.* 14: 6115-6128; and Li P., *et al.* (1987) Enzyme-linked Synthetic Oligonucleotide probes: Non-Radioactive Detection of Enterotoxigenic *Escherichia Coli* in Faeca Specimens. *Nuc. Acids Res.* 15: 5275-5287.]

Enzymes of interest as labels will primarily be hydrolases, such as phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescers include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol.

The oligonucleotide or polynucleotide acid probes of this invention can be included in a kit which can be used to rapidly determine the level of pre-rRNA in cells of a mycobacterial sample. The kit includes all components necessary to assay for the presence of the pre-rRNA. In the universal concept, the kit includes a stable preparation of labeled probes to pre-rRNA, hybridization solution in either dry or liquid form for the hybridization of target and probe polynucleotides, as well as a solution for washing and removing undesirable and nonduplexed polynucleotides, a substrate for detecting the labeled duplex, and optionally an instrument for the detection of the label.

A more specific embodiment of this invention embraces a kit that utilizes the concept of the sandwich assay. This kit would include a first component for the collection of samples from patients, vials for containment, and buffers for the dispersement and lysis of the sample. A second component would include media in either dry or liquid form for the hybridization of target and probe polynucleotides, as well as for the removal of undesirable and nonduplexed forms by washing. A third component includes a solid support upon which is fixed or to which is conjugated unlabeled nucleic acid probe(s) that is(are) complementary to a part of the precursor rRNA of the species of mycobacteria being tested. In the case of multiple target analysis more than one capture

BNSDOCID: <WO__9534574A1_I_>

cells exposed to the antimicrobial agent compared to mycobacterial cells not exposed to the antimicrobial agent.

Whether pre-rRNA levels increase or decrease in response to the antimicrobial agent will depend upon the step in the mycobacterial rRNA processing pathway that is inhibited by the particular agent. For example, rifampin works by inhibiting pre-rRNA synthesis while allowing processing to proceed. Thus, rifampin causes a complete or near-complete depletion of pre-rRNA in susceptible cells.

Chloramphenicol, kanamycin, and other translation inhibitors have the opposite effect. Chloramphenicol inhibits pre-rRNA processing without affecting synthesis. Therefore, pre-rRNA copy number increases in chloramphenicol-sensitive cells in the presence of chloramphenicol. Provided that appropriate quantitative controls are conducted to measure pre-rRNA copy number relative to cell number, one can use the methods described herein to determine chloramphenicol resistance or sensitivity.

Briefly, this procedure is performed as follows. First, the sample is decontaminated by standard methods [Heifets (1988) *Ann Rev. Respir. Dis.* 137: 1217-1222]. Next, a broth culture tube containing antibiotics is inoculated with the sample, as are control tubes that contain no antibiotic. The tubes are incubated for a period sufficient to allow the drug to act on pre-rRNA copy number. This period can be as short as a day or less. A sample is removed and the cells are lysed using the methods described herein. The amount of pre-rRNA is then determined. If desired, one can extract the nucleic acids, after which an amplification step can be employed. The amplification step, as described above, permits an even shorter culture period.

Adjustment of antibiotic challenge conditions to allow assessment of pre-rRNA response to antibiotics which do not directly affect RNA synthesis.

Our invention as described above and in examples to follow is particularly useful for assessing susceptibility of slow-growing mycobacteria to rifampicin, a front-line

BNSDOCID: <WO___9534574A1_I_>

more key nutrient. After approximately one day of incubation in this medium to allow depletion of pre-rRNA, the cells are exposed to the test antibiotic. A control culture not exposed to the antibiotic is run in parallel. Soon after antibiotic addition, the limiting nutrients are restored. Limiting nutrients that would supply nitrogen are representative of the nutrients which would deplete pre-RNA depletion. After another day of incubation, cells are lysed and assayed for pre-rRNA as described above and in examples below. Strains which are susceptible to the inhibitory effects of the antibiotic will display little or no replenishment of pre-rRNA relative to the control culture, whereas resistant strains will be indistinguishable from the control culture. This procedure lengthens the time course of the assay by a day or two, but enables clinicians to use our invention to test mycobacterial susceptiblity to virtually any antibiotic.

Alternative pre-treatments to deplete pre-rRNA prior to antibiotic challenge include the use of chemical agents that reversibly inhibit RNA synthesis in bacteria, such as N-thrichloromethylthio-4-cycloheximide (Luo and Lewis, *Biochem. Pharmocol.* **44**:2251-2258, 1992). The nature and composition of the pre-treatment is not important to this example, provided that it depletes pre-rRNA levels in mycobacterial cells, and that its effects can be reversed to allow measurable replenishment pre-rRNA upon its removal or cessation.

Drug Discovery

The present invention also provides a means for discovery of new anti-mycobacterial drugs. Prior to the instant invention, the slow growth rate of mycobacteria forced researchers to culture cells for several weeks in order to observe the effects of potential drugs. Because of this long incubation period, the potential drug compounds often degraded. Only unusually stable compounds could be found to have efficacy.

Using the claimed methods, drug developers can now identify compounds that are more effective, but less stable, than those previously identified. In less than one day, one

BNSDOCID: <WO___9534574A1_I_>

temperature and either assayed immediately or stored at -20°C for later analysis. Frozen lysates were thawed at room temperature, heated to 85°C, and allowed to return to room temperature before use.

DNA probe synthesis. We synthesized DNA probes using standard phosphoramidite chemistry on either an Applied Biosystems 380B or a Milligen 7500 automated DNA synthesizer, and purified as previously described [Van Ness *et al.* [(1991) *Nucl. Acids Res.* 19: 3345-3350; Van Ness and Chen (1991) *Nucl. Acids Res.* 19: 5143-5151].

Slot blot hybridization assays. Slot blot hybridization assays were carried out as described [Cangelosi *et al.* (1993) *Molecular and Cellular Probes* 8: 73-80; Dix *et al.* (1990) *J. Clin. Microbiol.* 28: 319-323]. Briefly, we extracted nucleic acids from 100 µl of *E. coli* lysates by phenol/chloroform extraction followed by ethanol precipitation. We then applied the nucleic acids to Nytran filters using a slot blot apparatus. We end-labeled DNA oligonucleotide probes with ^{32}P using polynucleotide kinase and hybridized the probe to the filters for 6-12 hours, as described in Cangelosi *et al.*, *supra.*, and Dix *et al.*, *supra.* After washing, we exposed the filters to Kodak X-Omat autoradiography film.

Chemiluminescent DNA probe sandwich assays. DNA probe sandwich assays consisted of capture probes tethered to activated nylon beads, biotinylated signal probes, washes, a streptavidin/alkaline phosphatase conjugate solution, a chemiluminescent substrate (Lumigen, Inc., Detroit, MI), and a Luminoskan luminometer (Labsystems, Finland). Assays were carried out in multi-well tissue culture plates using reagents and procedures described by Van Ness *et al.* (1991), *supra.* and Van Ness and Chen (1991), *supra.* 5'-Hexylamine-tailed oligonucleotides were biotinylated (signal probes) or activated and attached to nylon beads (capture probes) as described [Van Ness *et al.* (1991), *supra.*

BNSDOCID: <WO___9534574A1_I_>

Table 5: DNA probes. Negative (-) position numbers are upstream of the 5' 16S rRNA terminus and positive (+) position numbers are downstream of the 3' 16S rRNA terminus. Other positions numbers are within the mature 16S rRNA, starting from the 5' end.

PROBE	TARGET POSITION	SEQUENCE
EC012	+3 to +31	5'-GTGTGAGCACTACAAAGTACGCTTCTTTAA-3'
EC013	1538 to +25	5'-GCACTACAAAGTACGCTTCTTTAAGGTAAG-3'
EC014	-102 to -73	5'-ACTTGGTATTCATTTTTCGTCTTGCGACG-3'
EC016	456 to 475	5'-GCAAAGGTATTAACTTTACTCCCTTCCTCC-3'
EC020	179 to 206	5'-GTCCCCCTCTTTGGTCTTGCGACGTTAT-3'
UP042	1390 to 1409	5'-TGACGGGCGGTGTGTACAA-3'

Rate of pre-rRNA response to rifampin treatment. We used a slot blot hybridization assay to examine the rate of pre-rRNA decay in rifampin-treated cells. Cultures of rifampin-sensitive and rifampin-resistant *E. coli* strains 11775 and 11775-R1 in exponential growth were challenged by addition of rifampin to a final concentration of 20 μg/ml. Control cultures were treated with an equivalent volume of dimethyl sulfoxide, the solvent used to deliver rifampin to the test cultures. One hundred microliter were taken and lysed immediately (within 3 minutes) after challenge, and at various time points thereafter.

We extracted the nucleic acids from the lysates and applied them to Nytran filters. We probed the filters with radiolabeled probe EC013, which is specific for the 3' tail of the pre-16S rRNA. Replicate filters were hybridized with EC016, which detects both precursor and mature rRNA. Within 45 minutes of rifampin addition, precursor rRNA became undetectable by EC013 in rifampin-sensitive cells but not in rifampin-resistant cells (Fig. 1). Precursor rRNA remained detectable in untreated cultures of both strains. Probe EC016, which hybridizes to mature rRNA, could not distinguish resistant and sensitive cultures over the course of the experiment, as expected due to the bacteriostatic action of rifampin and the stability of mature rRNA.

BNSDOCID: <WO___9534574A1_I_>

resistant) with rifampin as described above. After a 90 minute incubation, we assayed for pre-rRNA.

In the absence of rifampin, cultures that we started with an initial inoculum of 100% sensitive cells were indistinguishable from cultures started from an initial inoculum of 1%, 10%, or 100% resistant cells (Fig. 2). When treated with rifampin, the culture containing 100% sensitive cells gave a very low signal, and cultures containing progressively higher percentages of resistant cells gave progressively stronger signals. Significantly, cultures containing as few as 1% resistant cells were reproducibly distinguishable from the 100% sensitive cultures. This result indicates that the magnitude of the response of pre-rRNA to rifampin challenge in susceptible *E. coli* cells is sufficient to make it a useful marker for sensitivity to the drug.

Example 2: Lysis of mycobacteria using an enzymatic treatment

The mycobacteria are cultured on modified DuBos medium to $1x10^{11}$ cells. The cells are then harvested by low speed centrifugation and the pellet resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. The cells are then digested by lysozyme at a final concentration of 10 mg/ml and Protease K at 0.1 mg/ml. The mixture is then incubated for 30 minutes at 37°C.

The treated cells are then centrifuged and the pellet resuspended in 8 mls of lysis buffer consisting of 100mM Tris, 10mM EDTA, 2% Sarcosyl, 0.5% SDS, and 0.1% PROCLIN™ at pH 7.5. The lysis buffer is then heated to 85°C for five minutes. The cell contents are now lysed and are ready for hybridization with mycobacterium-specific nucleic acid probes. The above lysis process can be conducted in about 45 minutes.

BNSDOCID: <WO___9534574A1_I_>

Ness and Chen (1991) *supra.*]. Probes were end-labeled with ^{32}P using polynucleotide kinase and hybridized with the blotted nucleic acid, using standard techniques [Dix *et al.* (1990) *supra.*]. Hybridization was measured by autoradiography.

RESULTS

Probe MTB030 hybridized strongly to the nucleic acids isolated from *M. tuberculosis*. However, probe MTB530 hybridized only weakly. This result indicates that the nucleic acids extracted from these cells contains a high copy number of pre-rRNA molecules (detected by MTB030), and a much lower copy number of chromosomal DNA molecules (detected by MTB530).

A drastic decrease in the ratio of pre-rRNA to DNA will be observed in sensitive cells treated with antibiotics that deplete pre-rRNA, such as rifampin. If the cells are resistant to the antibiotic, this drastic decrease will not be observed. Therefore, this procedure can be used to quickly assess bacterial response to rifampin and other compounds that inhibit RNA synthesis or processing, and to detect strains that are resistant to these compounds.

Example 4: Detection of *M. tuberculosis* pre-rRNA using a chemiluminescent sandwich assay

Importantly for a rapid and convenient assay for pre-rRNA, denaturing of the pre-rRNA is not necessary. The assay incorporates oligonucleotide probes that selectively hybridize to single-stranded regions of the pre-rRNA.

Cells were cultured and lysed by the enzymatic method described in Table 1c. DNA probe MTB030 (5'-CCCAAACACTCCCTTTGGAAAAGGG-3'), which is specific for a single-stranded region of the 5' tail of the pre-16S rRNA, was synthesized and labeled with biotin as described [Van Ness *et al.* (1990) *supra.*]. Probe MTB002 (5'-GTATCTCCGAAGAGACCTT-TCCGTTCG-3'), which is specific for a single-stranded region present within the mature 16S rRNA, was synthesized with a 5' amine tail and tethered to nylon beads as described [Van Ness and Chen (1991) *supra.*; Van Ness *et al.* (1991) *supra.*]. We

BNSDOCID: <WO___9534574A1_I_>

RESULTS

When MTB030 was used as a signal probe, a significant signal was observed (Fig. 4). For comparative purposes, we ran a parallel assay using MTB034 (5'-GGCCAAAAATAACAACAAAAATGTGAAACC-3') as a signal probe. This probe recognizes a different portion of the 5' pre-16S rRNA tail, starting 96 nucleotides upstream of the mature rRNA 5' terminus. No signal was observed using MTB034, suggesting that the region of the pre-rRNA to which MTB034 binds is double-stranded under the conditions employed in this assay. Therefore, access of MTB034 to the target is impeded.

This Example demonstrates the usefulness of probes that target single-stranded regions of the pre-rRNA. A partial list of target sequences in *M. tuberculosis* pre-rRNA that function well under the non-denaturing conditions employed in this experiment is presented in Table 2. Target sequences for pre-rRNA of other mycobacterial species are presented in Table 3.

The probes listed in Tables 2 to 4 provide strong signals in combination with a variety of different capture probes for mature rRNA sequences. Positive results are also obtained when the sandwich is reversed, such that the capture probe is specific for pre-rRNA tail sequences, and the signal probe hybridizes to sequences within the mature rRNA. Some of these oligonucleotide probes hybridized to regions not thought to be single stranded, according to published models for mycobacterial pre-rRNA secondary structure based upon DNA sequence analysis [Kempsell *et al.* (1992) *supra.*; Ji *et al.* (1994), *supra.*]. Thus, the binding of these probes under denaturing conditions was not expected.

Also unexpected was the good performance of probes MTB015 and MTB016, which target the 5' tail of the pre-23S rRNA. When used in combination with a capture probe specific for single-stranded regions of the mature 23S subunit, these probes gave the same or better sensitivity than probes for the pre-16S rRNA. This was unexpected in light of published data on processing of the 23S rRNA in other bacteria. For example, it has been reported that pre-23S rRNA is much less abundant

BNSDOCID: <WO___9534574A1_I_>

in all solutions used in the lysis procedure to prevent processing of pre-rRNA during lysis. We detected pre-rRNA using a chemiluminescent sandwich assay similar to that described in Example 4. We used as a capture probe MSM001 (5'CGGCTCCCTCCACAAGGGTTAGGCCACC3'), which recognizes an open region within the mature rRNA of *M. smegmatis*,. The signal probe was MSM008 (5'TCACACCCTCCCCAACGGA3'), which recognizes an open region in the 3' pre-16S precursor tail of this organism.

The results of this experiment are shown in Table 6. Although the brief antibiotic treatment had no effect on culture turbidity or other visible aspects of culture density, it had a measurable and reproducible effect on pre-rRNA concentration as measured by the chemiluminescent sandwich assay.

Table 6. Effects of antibiotic treatment on pre-rRNA copy number in *M. smegmatis*

Treatment	Culture density	pre-rRNA/ml (luminometer units)
None	$3X10^8$ cells/ml	6.0
Kanamycin	$3X10^8$ cells/ml	22.5
Chloramphenicol	$3X10^8$ cells/ml	41.9

Using *E. coli* as a model, we observed similar 5- to 7-fold increases in pre-rRNA copy number in response to chloramphenicol and kanamycin treatment (data not shown). The data presented here support the assumption that processing pathways for the mycobacterial and *E. coli* 16S rRNA are similar.

Rifampin treatment can be expected to have an opposite, and even more dramatic, effect compared to kanamycin. In Example 1, we demonstrated that rifampin causes more than a 100-fold decrease in *E. coli* pre-rRNA copy number within one generation time of exposure to the drug. *M. tuberculosis*, is also very sensitive to rifampin [Heifets (1988) *Am. Rev. Respir. Dis.* 137: 1217-1222]. Therefore, pre-rRNA in rifampin-sensitive *M. tuberculosis* will also decrease in response to rifampin. Similarly, other antimicrobial agents will cause similar effects by directly or

BNSDOCID: <WO___9534574A1_I_>

effects become visible. As illustrated in the present example, measurements of pre-rRNA content can shorten this time considerably. By hybridizing extracted *M. tuberculosis* H37Ra nucleic acid to a radiolabeled oligonucleotide probe specific for *M. tuberculosis* pre-16S rRNA tail sequences, we detected clear responses to rifampicin and ciprofloxacin within 3 hours and 48 hours, respectively, of *in vitro* antibiotic exposure.

M. tuberculosis strain H37Ra (ATCC 25177) was cultured on Dubos broth with albumin enrichment (Difco Laboratories, Detroit, MI) to an optical density at 600 nanometers (OD_{600}) of 0.2-0.4. Cells were diluted 5-10 fold in 75 ml of fresh broth (final OD600 = 0.04-0.05) in fluted 250 ml culture flasks. Rifampicin or ciprofloxacin (Sigma Chemical Co., St. Louis, MO) were added to a final concentration of 5 ug/ml, and control cultures were left untreated. Cultures were incubated with gentle agitation under air at 37°C, and additional samples were taken after approximately 3 hours, 7 hours, 24 hours, and 48 hours.

Immediately prior to antibiotic addition and at several time points thereafter, culture optical density was measured and samples were taken as follows. Ten milliliters of each culture was centrifuged at low speed to pellet the cells, and the pellets were resuspended in approximately 1/6 volume of TE buffer. Guanidinium lysates were prepared as described in Table 1c "Rapid enzymatic method", except that proteinase K was used at 0.01 mg/ml, and both enzymes were added to TE prior to resuspending the cells. The lysates were stored frozen until use.

Nucleic acid was extracted from 0.2 ml of each lysate by phenol-chloroform extraction, and applied to 0.22 micron Magna NT membrane filters (MSI, Westboro, MA) using a slot blot apparatus as described by Moncla et al. (*J. Clin. Microbiol.* **28**:324-327, 1990). For the purposes of this demonstrative experiment, each sample was divided between two filters which were in turn cut in half before hybridization to probes, resulting in four replicate blotted samples per 0.2 ml of lysate. This corresponded to the application of nucleic

3NSDOCID: <WO 9534574A1 I >

In all experiments of this type, antibiotic treatment decreased pre-rRNA to barely detectable levels which then remained constant over time. This "background" may have been due to residual pre-rRNA, or to hybridization of the probe to the chromosomal *rrn* operon DNA coding for the pre-rRNA. To test the latter possibility with the greatest possible sensitivity, parallel cultures of *M. tuberculosis* were treated with rifampicin or left untreated as described above, except that the experiment was carried out at approximately five times the cell concentration. Replicate filters were hybridized to MTB030 and its complement, MTB030r, which is specific for the coding strand of the *rrn* operon DNA. After extended autoradiography to clearly reveal background signal, MTB030 signal did not measurably exceed MTB030r signal in nucleic acid extracted from rifampicin-treated cells (Figure 6). Similar results were obtained when replicates of the filters shown in Figure 5 were hybridized to MTB030r and overexposed (data not shown). These data suggest that pre-rRNA copy number in rifampicin-treated cells does not measurably exceed that of the DNA encoding it.

Our results show that pre-rRNA levels respond extremely rapidly to certain antibiotics, making pre-rRNA an attractive physiological indicator of antibiotic susceptibility in the slow-growing mycobacteria. The "background" contributed by hybridization of probes to DNA *rrn* operon sequences could be greatly reduced or eliminated by hybridizing to DNA probes in the presence of chaotropic trichloroacetate salts, which favor DNA:RNA interactions over DNA:DNA interactions (Van Ness and Chen, *Nucl. Acids Res.* **19**:5143-5151, 1991). Alternatively, the DNA could be selectively degraded using deoxyribonucleases.

It will be apparent to one skilled in the art that sensitivity of this assay for pre-rRNA could be improved somewhat by using longer probes and more sensitive labeling methods, and to a much greater extent by using *in vitro* RNA amplification techniques such as NASBA or reverse transcriptase PCR, as described in Example 6. Such amplification would allow clinicians to apply our invention

BNSDOCID: <WO_ _9534574A1_I_>

WHAT IS CLAIMED IS:

1. A method of lysing mycobacterial cells, the method comprising the steps of:

(a) treating the cells by enzymatic degradation using both lysozyme and protease until their cell walls are rendered porous to expose their cell membranes making the cells susceptible to lysis by steps (b) and (c);

(b) contacting the treated cells with a combination of a magnesium chelator, a nonionic detergent and an anionic detergent; and

(c) heating the cells to between about between 75°-99°C until the mycobacterial cells are lysed.

2. A method of claim 1 wherein the chelator is selected from the group consisting of: ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis(β-amino-ethyl ether)-tetraacetic acid (EGTA), and ethylene diimino dibutyric acid (EDBA).

3. A method of claim 1 wherein the nonionic detergent is selected from the group consisting of: sarcosyl, Triton X, Brij, Tween, and NP-40.

4. A method of claim 1 wherein the anionic detergent is selected from the group consisting of: dodecylsulfate, laurylsulfate, and deoxycholic acid.

5. A method of claim 1 wherein the treatment is a combination of lysozyme and proteinase K.

6. A method of claim 1 wherein the heating step is above 85°C and has a duration of 5 minutes or greater.

7. A method for detecting pre-ribosomal RNA (pre-rRNA) in cells of a mycobacterial sample, the method comprising the steps of:

BNSDOCID: <WO__9534574A1_I_>

14. A method of claim 13 wherein the pre-rRNA is amplified by 3SR.

15. A method of claim 13 wherein the pre-rRNA is amplified by culturing the mycobacterial sample in a medium containing chloramphenicol.

16. A method of claim 8 wherein the mycobacterial sample comprises cells of at least one of the group consisting of *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium habana*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium marinum*, *Mycobacterium simiae*, and *Mycobacterium intracellulare*.

17. A method of claim 16 wherein the oligonucleotide probe selectively hybridizes to a mycobacterial pre-rRNA region having a nucleotide sequence or a specific subsequence selected from the group consisting of:

for *M. tuberculosis*:

1) 5'AAGGAGCACCAGCAAAACGCCCCCC3';
2) 5'GGGUGCAUGACAACAAAGUUGGCCA3';
3) 5'GGCCACCACCACACUGUUGGGUCCUGAGGC3';
4) 5'UUGCGAGCAUCAAUGGAUACGCUGCC3';
5) 5'ACGCUGCCGGCUAGCGGUGGCGUG3';
6) 5'UUCUUUGUGCAAUAUUCUUUGGUUUUUGUUGUG3';
7) 5'UUGUCGGGGGGCGUGGCCGUUUG3'; and
8) 5'CCCUUUUCCAAAGGGAGUGUUUGGG3';

for *M. bovis*:

1) 5'AAGGAGCACCACGAAAACGCCCC3';
2) 5'GGGUGCAUGACAACAAAGUUGGCCA3';
3) 5'GGCCACCACCACACUGUUGGGUCCUGAGGC3';
4) 5'UUGCGAGCAUCAAUGGAUACGCUGCC3';
5) 5'GGCUAGCGGUGGCGUG3'; and
6) 5'UUCUUUGUGCAAUAUUCUUUGGUUUUUGUUGUG3';

for *M. avium*:

1) 5'AAGGAGCACCACGAAAAGCACCCCC3';
2) 5'GGGUGCGCAACAGCAAAUGAUUGCCA3';

NSDOCID: <WO 9534574A1 I >

1) 5'UGUGUGGGUAUGGCAA3'; and
2) 5'CUGAUUUGAAAUUCACCUCGCUGCGCGAGGAGAU3';
for *M. marinum*:
1) 5'UGUGAGGGAGUAGUCGUU3'; and
2) 5'CUGAUUGCGAAUUCACCUCGUUAUCGAGGGGUU3';
for *M. habana*:
1) 5'UGUGUAGGUAUGGUCGU3'; and
2) 5'CAGAUUAUCUCUGAUUCGAAUCCACCUCGUUGAUCGAGGAGAU3'.

18. A method for determining whether cells of a mycobacterial sample are sensitive to an antimicrobial agent, the method comprising the steps of:

(a) culturing the mycobacterial cells in the presence of the antimicrobial agent;

(b) treating the cells by enzymatic or mechanical means to expose the cell membrane to lysis reagents, and contacting the cells with a lysis reagent under conditions that release but do not degrade pre-rRNA from the mycobacterial cells; and

(c) detecting the pre-rRNA using an oligonucleotide probe;

wherein sensitivity to the antimicrobial agent is indicated by an increase or a decrease in pre-rRNA levels for mycobacterial cells exposed to the antimicrobial agent compared to mycobacterial cells not exposed to the antimicrobial agent.

19. A method of claim 18 wherein the antimicrobial agent is rifampin.

20. A device for detecting pre-rRNA in a mycobacterial sample, the device comprising an oligonucleotide probe immobilized on a solid support, wherein the oligonucleotide probe is between about 10 to 100 nucleotides in length and is capable of selectively hybridizing, under hybridizing conditions, to a region of a mycobacterial pre-

BNSDOCID: <WO___9534574A1_I_>

8) 5'CUUGAUUUGAAAUUCACCUCGCUGCGCGAGGAGAU3';

for *M. lufu*:

1) 5'AAGGAGCACCACGAAAAGCUACCC3';

2) 5'GGGUGCACAACAGCAAAUGAUUGCCA3';

3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';

4) 5'UUGCGAGCUACUAGAUGAACGCGUAGU3';

5) 5'CGCGUAGUCCUUGGGGCUGACGAGUUC3';

6) 5'AUCGAAAUGUGUUAUUUCUUUUUUAACUCUUGUG3';

7) 5'UGUGUGGGUAUGGUUGU3'; and

8) 5'CUGAUUUGAAUUCACCUCGUUCUGCGAGGAGUU3';

for *M. intracellulare*:

1) 5'AAGGAGCACCACGAAAAGCACUCC3';

2) 5'GGGUGCACAACAGCAAAUGAUUGCCA3';

3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';

4) 5'UUGCGAGCAUCUAGAUGAGCGCAUAGU3';

5) 5'CGCAUAGUCCUUAGUGAUGCGUC3';

6) 5'GUCGAAAUGUGUAAUUUCUUCUUUGGUUUUUGUG3';

7) 5'UGUGUGGGUAUGGCAA3'; and

8) 5'CUGAUUUGAAAUUCACCUCGUUCAUCGAGGAGUU3';

for *M. leprae*:

1) 5'AAGGAGCACCACGAAAAACACUCUAA3';

2) 5'GGGUGCGCAACAGCAAAUAUCCA3';

3) 5'CCAGACACACUGUUGGGUCCUGAGGC3';

4) 5'UUGCGAGCAUCUAAAUGGAUGCGUUGUC3';

5) 5'GCGUUGUCAGUUAUGUAGUGGUGGCGU3';

6) 5'AUUCAUUGAAAAUGUGUAAUUUUCUUCUUUGGUUUUGUG3';

7) 5'UGUGUGUAGGUGUAGUUUAUUA3'; and

8) 5'CUAGAAAUUGAAAAUUUCGUCUAGUUAUUGAUGGAGUU3';

for *M. simiae*:

1) 5'AAGGAGCACCACGAGAAACACUCC3';

2) 5'GGGUGCACAACAACAGGCAAUCGCCA3'; and

3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';

for *M. paratuberculosis*:

1) 5'UGUGUGGGUAUGGCAA3'; and

2) 5'CUGAUUUGAAAUUCACCUCGCUGCGCGAGGAGAU3';

for *M. marinum*:

1) 5'UGUGAGGGAGUAGUCGUU3'; and

2) 5'CUGAUUGCGAAUUCACCUCGUUAUCGAGGGGUU3';

BNSDOCID: <WO 9534574A1 I >

1) 5'AAGGAGCACCACGAAAACGCCCC3';
2) 5'GGGUGCAUGACAACAAAGUUGGCCA3';
3) 5'GGCCACCACCACACUGUUGGGUCCUGAGGC3';
4) 5'UUGCGAGCAUCAAUGGAUACGCUGCC3';
5) 5'GGCUAGCGGUGGCGUG3'; and
6) 5'UUCUUUGUGCAAUAUUCUUUGGUUUUUGUUGUG3';

for *M. avium*:

1) 5'AAGGAGCACCACGAAAAGCACCCCC3';
2) 5'GGGUGCGCAACAGCAAAUGAUUGCCA3';
3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';
4) 5'UUGCGAGCAUCUAGAUGAGCGCAUGGU3';
5) 5'CGCAUGGUCUUCGUGGCCGGCGUUC3';
6) 5'AUCGAAAUGUGUAAUUUCUUUUUUAACUCUUGUG3';
7) 5'UGUGUGGGUAUGGCAA3'; and
8) 5'CUUGAUUUGAAAUUCACCUCGCUGCGCGAGGAGAU3';

for *M. lufu*:

1) 5'AAGGAGCACCACGAAAAGCUACCC3';
2) 5'GGGUGCACAACAGCAAAUGAUUGCCA3';
3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';
4) 5'UUGCGAGCUACUAGAUGAACGCGUAGU3';
5) 5'CGCGUAGUCCUUGGGGCUGACGAGUUC3';
6) 5'AUCGAAAUGUGUUAUUUCUUUUUUAACUCUUGUG3';
7) 5'UGUGUGGGUAUGGUUGU3'; and
8) 5'CUGAUUUGAAUUCACCUCGUUCUGCGAGGAGUU3';

for *M. intracellulare*:

1) 5'AAGGAGCACCACGAAAAGCACUCC3';
2) 5'GGGUGCACAACAGCAAAUGAUUGCCA3';
3) 5'GCCAGACACACUAUUGGGCCCUGAGAC3';
4) 5'UUGCGAGCAUCUAGAUGAGCGCAUAGU3';
5) 5'CGCAUAGUCCUUAGUGAUGCGUC3';
6) 5'GUCGAAAUGUGUAAUUUCUUCUUUGGUUUUUGUG3';
7) 5'UGUGUGGGUAUGGCAA3'; and
8) 5'CUGAUUUGAAAUUCACCUCGUUCAUCGAGGAGUU3';

for *M. leprae*:

1) 5'AAGGAGCACCACGAAAAACACUCUAA3';
2) 5'GGGUGCGCAACAGCAAAUAUCCA3';
3) 5'CCAGACACACUGUUGGGUCCUGAGGC3';
4) 5'UUGCGAGCAUCUAAAUGGAUGCGUUGUC3';

BNSDOCID: <WO 9534574A1 I >

29. A method of claim 28 wherein the cells are depleted of pre-RNA by limiting a nutrient.

30. A method of claim 28 wherein the cells are depleted of pre-RNA by exposing the cells to a second antibiotic compound which is other than the test compound and removing the second compound prior to contact with the test compound.

BNSDOCID: <WO___9534574A1_I_>

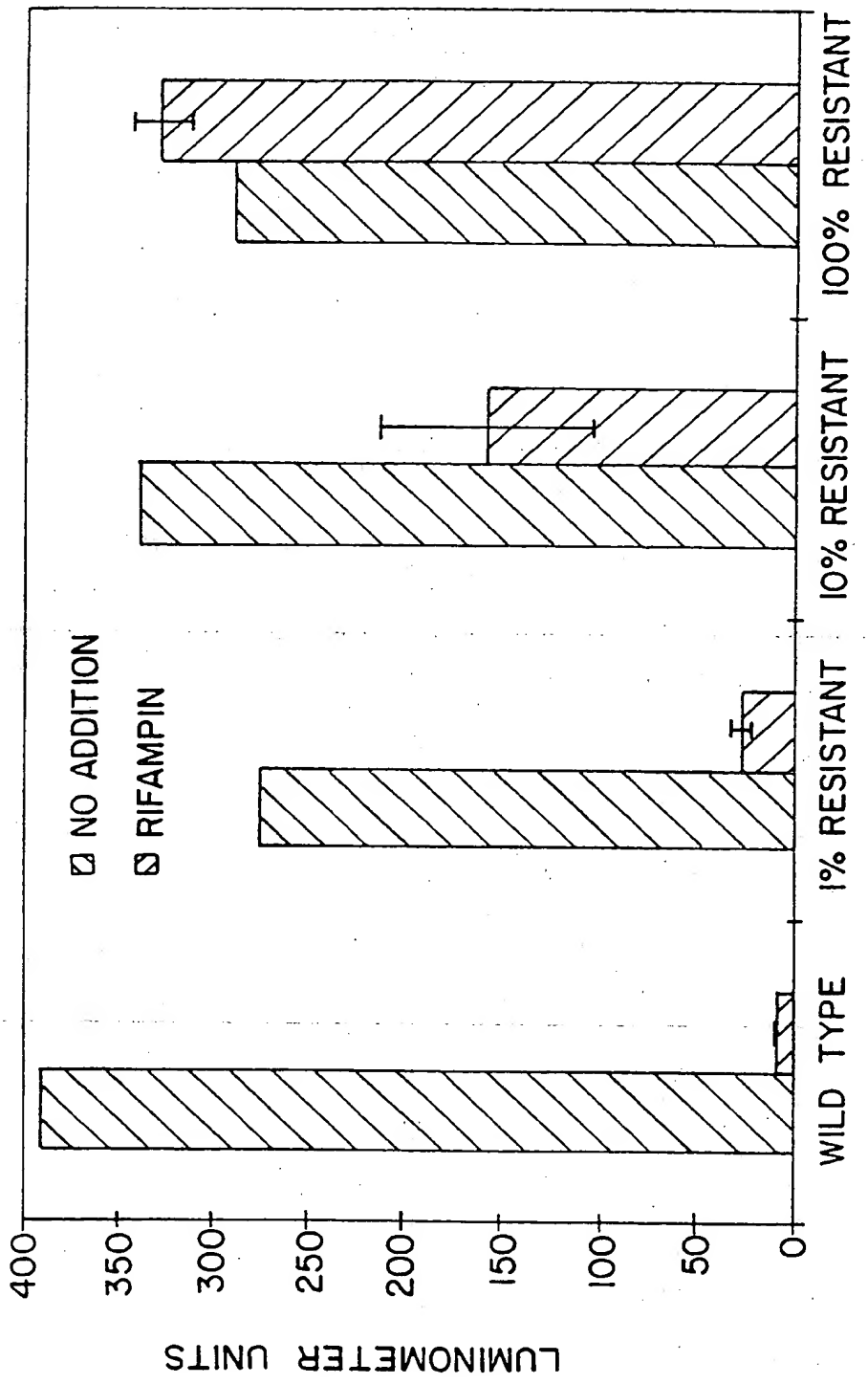


FIG. 2.

BNSDOCID: <WO 9534574A1 I >

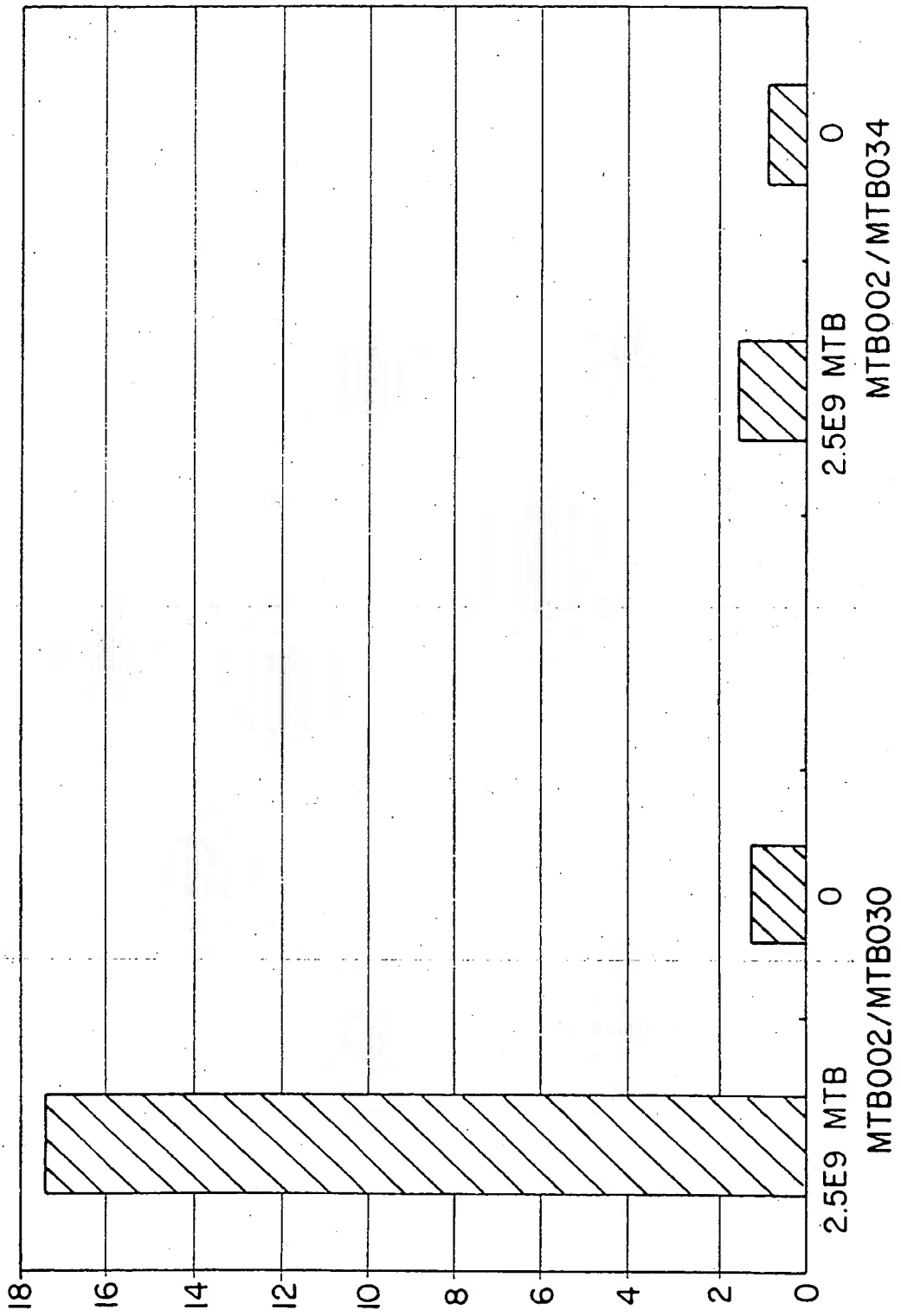


FIG. 4.

BNSDOCID: <WO___9534574A1_I_>

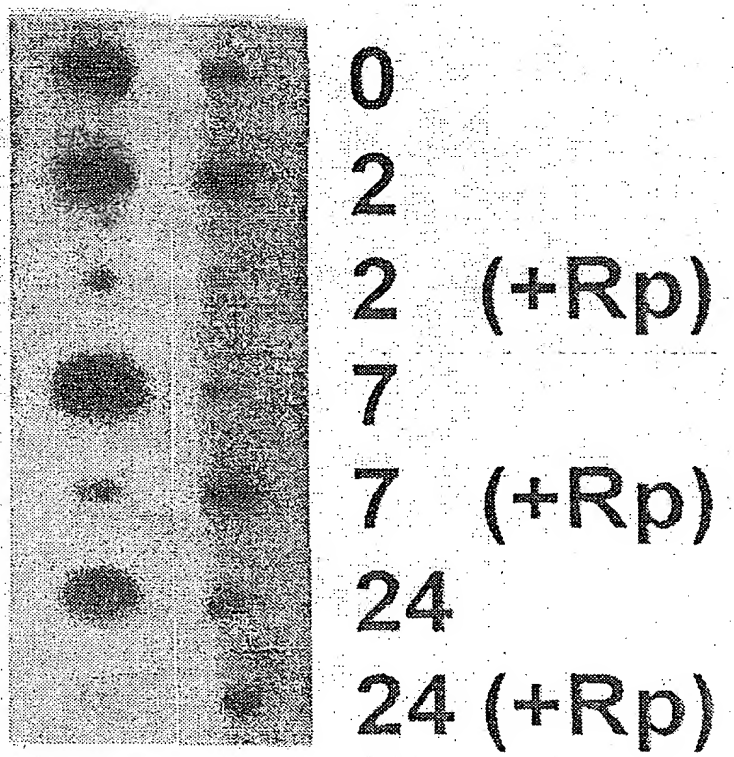


FIG. 6.

BNSDOCID: <WO 9534574A1 I >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07755

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Microbiology, Volume 138, Part 8, issued August 1992, Kempsell et al, "The Nucleotide Sequence of the Promoter, 16S rRNA and Spacer Region of the Ribosomal RNA Operon of Mycobacterium tuberculosis and Comparison with Mycobacterium leprae Precursor rRNA", pages 1717-1727, see page 1720, Figure 2.	7-30

BNSDOCID: <WO 9534574A1 I >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07755

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-6, drawn to a method of lysing mycobacterial cells, classified in Class 435, subclass 4.

II. Claims 7-30, drawn to method of detecting Mycobacteria by nucleic acid hybridization, method of determining Mycobacterial sensitivity to antimicrobial agent, oligonucleotide probes, device and kit for detecting Mycobacteria, classified in Class 435, subclasses 6, 810; and Class 536, subclass 24.32.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is drawn to methods of lysing Mycobacteria while Group II is drawn to methods, products, device and kit for the detection thereof. The inventions differ in that they require different reagents and experimental set-ups, e.g. lysing buffer in Group I, and oligonucleotide probes in Group II.

Regarding Group II, the Mycobacterial species (claims 16, 21) and nucleotide sequence of oligonucleotide probes (claims 17, 21, 22, 26) drawn in these claims do not relate to a single inventive concept under the same rule because they lack the same or corresponding special technical features: Each Mycobacteria species and oligonucleotide probes for the detection thereof are distinct in that the species are unique, and the nucleotide sequences to detect thereof are specific only to each species.

BNSDOCID: <WO 9534574A1 I >